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Title of the Invention:

CHIMERIC EMBRYOS AND ANIMALS CONTAINING HUMAN CELLS

COMMUNICATION

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Dear Sir:

Attached hereto for the Examiner's consideration are copies of two references referred to in the Remarks section of Applicant's amendment filed 29 April 2003 in the above-identified matter, specifically

Gilbert's *Developmental Biology* (Sinauer, 1997), pages 187 and 189 (cited at page 9 of the Remarks)

An Introduction to Embryology, Fourth Ed. (1975), Balinsky, B.I., W.B. Saunders Co., Philadelphia, PA (cited at page 13 of the Remarks)

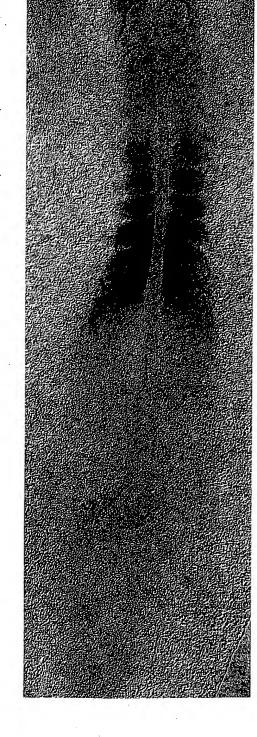
Although no specific passage in Balinsky was mentioned, Applicant submits a copy of Chapter I thereof as most relevant to the subject matter at hand.

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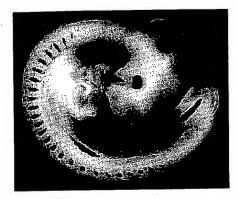
Developmental Biology

FIFTH EDITION

Scott F. Gilbert
Swarthmore College



SINAUER ASSOCIATES, INC.
PUBLISHERS
Sunderland, Massachusetts



The cover

COVER PHOTOGRAPH: The mRNA for Fibroblast Growth Factor-8 can be detected by wholemount in situ hybridization using chemically labeled RNA that is complementary to this message. In this 3-day chick embryo, the *Fgf8* message is found in the most distal ectoderm of the limb buds, in the boundary between the midbrain and hindbrain, in the somites, in the branchial arches of the neck, and in the developing tail. FGF8 is important for several developmental processes, and it plays critical roles in the outgrowth of the limbs and the patterning of the developing brain. Chapters 3, 7 and 18. (Photograph courtesy of E. Laufer, C.-Y. Yeo and C. Tabin.)

BACK COVER PHOTOGRAPH: Photograph of a Day 20–21 chicken embryo at the pipping and prehatching stage. Note the prominent peridermal covering at the tip of the beak (egg-tooth), used by the chick to make holes in the eggshell, which has become thinner and more brittle as a consequence of mineral utilization by the embryo for its growing skeleton. This developmental stage marks the transition of the embryo into an air-breathing chick. Chapters 1 and 5. (Photograph from the *International Poultry Journal*, courtesy of R. Tuan.)

The title pages

LEFT PAGE: Gene expression generates boundaries in *Drosophila* imaginal discs. The large and small discs within the fly larva form the adult wing and haltere, respectively. At this stage, Apterous protein (red) is expressed only in the dorsal compartments; the Cubitus interruptus protein (blue) marks the anterior (but not the posterior) compartments (a line forming this boundary can be seen). The green staining (from the Vestigial protein) in the interior demarcates the boundary between the free limb and the hinge linking it to the thoracic wall. Chapter 19. (Photograph courtesy of J. Williams, S. Paddock and S. Carroll.)

RIGHT PAGE: Expression of the *paraxis* gene in the 6-somite chick embryo. Wholemount in situ hybridization using a digoxygenin-labeled RNA complementary to a portion of the chick *paraxis* message shows the expression of this gene during somite formation. The Paraxis protein is important in establishing the structure of these mesodermal clusters. Chapters 2 and 9. (Photographic montage courtesy of R. Tuan.)

Developmental Biology, FIFTH EDITION

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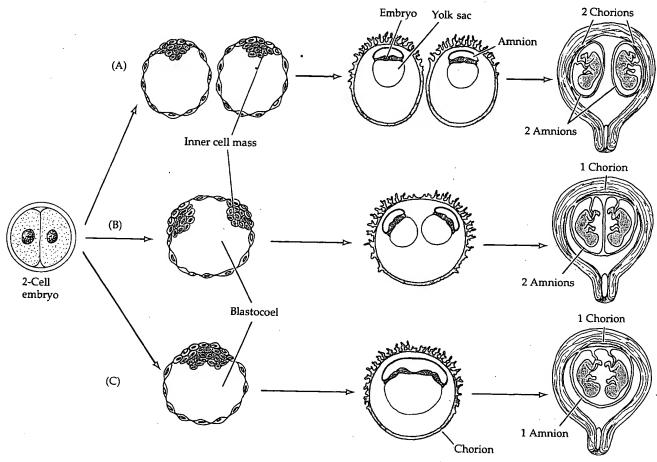


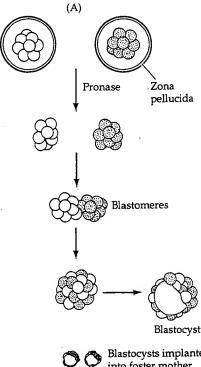
Figure 5.27 Diagram showing the timing of human monozygotic twinning with relation to extraembryonic membranes. (A) Splitting occurs before the formation of the trophectoderm, so each twin has its own chorion and amnion. (B) Splitting occurs after trophectoderm formation but before amnion formation, resulting in twins having individual amniotic sacs but sharing one chorion. (C) Splitting after amnion formation leads to twins in one amniotic sac and a single chorion. (After Langman, 1981).

embryos to form one chimeric mouse rather than twins, triplets, or a multiheaded monster. Chimeric mice are the result of two or more early-cleavage (usually 4- or 8-cell) embryos that have been artificially aggregated to form a composite embryo. As shown in Figure 5.28A, the zonae pellucidae of two genetically different embryos are removed and the embryos brought together to form a common blastocyst. These prepared blastocysts are implanted into the uterus of the foster mother. When they are born, the chimeric offspring have some cells from each embryo. This is readily seen when the aggregated blastomeres come from mouse strains that differ in their coat colors. When blas-

tomeres from white and black strains are aggregated, the result is commonly a mouse with black and white bands (Figure 5.28B). There is even evidence (de la Chappelle et al., 1974; Mayr et al., 1979) that human embryos can form chimeras. These individuals have two genetically different cell types (XX and XY) within the same body, each with its own set of genetically defined characteristics. The simplest explanation for such a phenomenon is that these individuals resulted from the aggregation of two embryos, one male and one female, that were developing at the same time. If this explanation were correct, then two fraternal twins fused to create a single composite individual.

Markert and Petters (1978) have shown that three early 8-cell embryos can unite to form a common compacted morula (Figure 5.29) and that the resulting mouse can have the coat colors of the three different strains (Plate 21). Moreover, they showed that each of the three embryos gave rise to precursors of the gametes. When a chimeric (black/brown/white) female mouse was mated to a white-furred (recessive) male, the offspring were each of the three colors.

According to our observations of twin formation and chimeric mice, each blastomere of the inner cell mass should be able to produce any cell of the body. This hypothesis has been confirmed, and it will have very important consequences



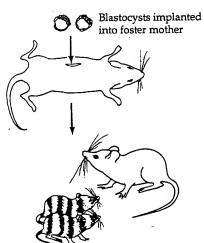
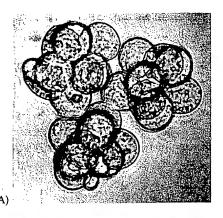
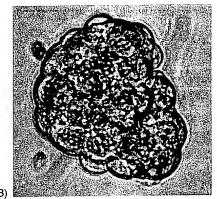


Figure 5.28 Production of chimeric mice. (A) The experimental procedures used to produce chimeric mice. Early 8-cell embryos of genetically distinct mice (here, those with coat-color differences) are isolated from mouse oviducts and brought together after their zonae are removed by proteolytic enzymes. The cells form a composite blastocyst, which is implanted into the uterus of a foster mother. (B) An adult chimeric mouse showing contributions from the pigmented (black) and unpigmented (white) embryos. (Photograph courtesy of B. Mintz.)

in studying mammalian development. When inner mass cells are isolated and grown under certain conditions, they remain undifferentiated and continue to divide in culture (Evans and Kaufman, 1981; Martin, 1981). These cells are called embryonic stem cells (ES cells). As shown in Chapter 2, these cells can be altered in the petri dish. Cloned genes can be inserted into their nuclei, or the existing genes can be mutated. When these ES cells are injected into blastocysts of another mouse embryo, the ES cells can integrate into the host inner cell mass. The resulting embryo has cells coming from both the host and the donor tissue. This technique has become extremely important in determining the function of genes during mammalian development.





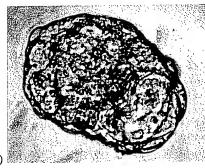
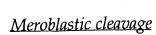


Figure 5.29 Aggregation and compaction of three 8-cell mouse embryos to form a single compacted morula. Cells from three different embryos (A) are aggregated together to form a morula (B), which undergoes compaction to form a single blastocyst (C). The resulting chimeric mouse is shown in Plate 21. (From Markert and Petters, 1978 courtesy of C. Markert.)



As mentioned earlier, yolk concentration plays an important role in cell cleavage. Nowhere is this more apparent than in the meroblastic cleavage types. Here, the large concentrations of yolk prohibit cleavage in all but a small portion of the egg cytoplasm. In discoidal cleavage, cell division is limited to a small disc of yolk-free cytoplasm atop a mound of yolk; in superficial cleavage, the centrally located yolk permits cleavage only along the peripheral rim of the egg.

Discoidal Cleavage

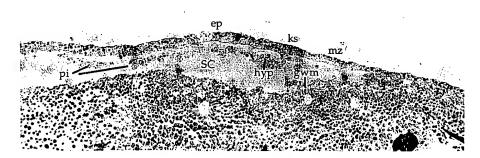
Discoidal cleavage is characteristic of birds, fishes, and reptiles.

BIRDS. Figure 5.30 shows the cleavage of an avian egg. The bulk of the oocyte is taken over by the yolk, allowing cleavage to occur only in the blastodisc, a region of active cytoplasm about 2-3 mm in diameter at the animal pole of the egg. Because these cleavages do not extend into the yolky cytoplasm, the early-cleavage cells are actually continuous at their bases. The first cleavage furrow appears centrally in the blastodisc, and other cleavages follow to create a single-layered blastoderm. At first, this cellular layer is incomplete, as the cells are still continuous with underlying yolk. Thereafter, equatorial and vertical cleavages divide the blastoderm into a tissue five to six cell layers thick. These cells become linked together with tight junctions (Bellairs et al., 1975; Eyal-Giladi, 1991). Between the blastoderm and the yolk is a space called the subgerminal cavity. This space is created when the blastoderm cells absorb fluid from the albumin ("egg white") and secrete it between themselves and the yolk (New, 1956). At this stage, the deep cells in the center of the blastoderm are shed to create a one-cell-thick area pellucida. (The shed cells appear to die.) The peripheral ring of blastoderm cells that are not shed constitute the area opaca.

By the time a hen has laid an egg, the blastoderm contains some 60,000 cells. Some of these cells are delaminated into the subgerminal cavity to form a second layer (Figure 5.31). Thus, soon after laying, the chick egg contains two layers of cells: the upper epiblast and the lower hypoblast. Between them lies the blastocoel. We will detail the formation of the hypoblast in the next chapter.

FISHES. In recent years, the zebrafish, Danio rerio, has become a favorite organism of those who wish to study vertebrate development. These fish have large broods, breed all year, are easily maintained, have transparent embryos that develop outside the mother (an important feature for microscopy), and can be raised so that mutants can be readily screened and propagated. In addition, they develop rapidly, so that at 24 hours after fertilization, the embryo has formed most of its tissue and organ primordia and displays the characteristic tadpole-like form (see Granato and Nüsslein-Volhard, 1996; Langeland and Kimmel, 1997).

The yolky eggs of fishes develop similarly to those of birds, with cell division occurring only in the animal pole blastodisc. Scanning electron micrographs of fish egg cleavage show beautifully the incomplete nature of dis-



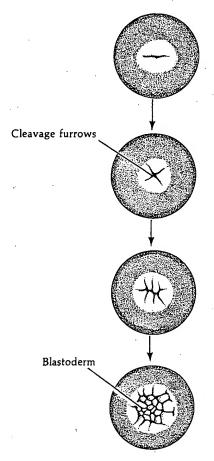


Figure 5.30 Discoidal cleavage in a chick egg, viewed from the animal pole. The cleavage furrows do not penetrate the yolk, and a blastoderm consisting of a single layer of cells is produced.

Figure 5.31

Formation of the two-layered chick embryo. This sagittal section of the embryo near the posterior margin shows an upper layer consisting of a central epiblast that trails into the cells of Koller's sickle (ks) and the posterior marginal zone (mz). Certain cells from the epiblast fall (delaminate) from the upper layer to form polyinvagination islands (pi) of 5 to 20 cells each. These cells will be joined by those hypoblast cells (hyp) migrating anteriorly from Koller's sickle to form the lower (hypoblastic) layer. (Sc is subgerminal cavity; gwm is germ wall margin.) (From Eyal-Giladi et al., 1992, courtesy of H. Eyal-Giladi.)

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Chapter 1

THE SCOPE OF EMBRYOLOGY AND ITS DEVELOPMENT AS A SCIENCE

1-1 ONTOGENETIC DEVELOPMENT AS THE SUBJECT MATTER OF EMBRYOLOGY

The aim of this book is to familiarize the student with the basic facts and problems of the science of embryology. The name "embryology" is somewhat misleading. Literally it means the study of embryos. The term "embryo" denotes the juvenile stage of an animal while it is contained in the egg (within the egg membranes) or in the maternal body. A young animal, once it has hatched from the egg or has been born, ceases to be an embryo and would escape from the sphere pertaining to the science of embryology if we were to keep strictly to the exact meaning of the word. Although birth or hatching from the egg is a very important occasion in the life of the animal, it must be admitted that the processes going on in the animal's body may not be profoundly different before and after the hatching from an egg, especially in some lower animals. It would be artificial to limit studies of the juvenile forms of animal life to the period before the animal is hatched from the egg or is born. It is customary, therefore, to study the life history of an animal as a whole and accordingly to interpret the scope of the science of embryology as the study of the development of animals.

The word "development" must be qualified in turn. In the sphere of biology with which we are concerned, the term "development" is used with two different meanings. It is used to denote the processes that are involved in the transformation of the fertilized egg, or some other rudiment derived from a parent organism, into a new adult individual. The term development may, however, also be applied legitimately to the gradual historical transformation of the forms of life, starting with simple forms which might have been the first to appear and leading to the contemporary diversity of organic life on our planet. Development of the first type may be distinguished as individual development or ontogenetic development. Development of the second type is the historical development of species or phylogenetic development. Phylogenetic development is often referred to as evolutionary development or simply evolution. Accordingly, we will define embryology as the study of the ontogenetic development of organisms. In this book we will be dealing only with the ontogenetic development of multicellular animals, the Metazoa.

In multicellular animals, the typical and most widespread form of ontogenetic development is the type occurring in sexual reproduction. In sexual reproduction new individuals are produced by special generative cells or gametes. These cells differ essentially from other cells of the animal, in that they go through the process of maturation or meiosis, as a result of which they lose half of their chromosomes and become

haploid, whereas all other cells of the parent individual, the somatic cells, are, as a rule, diploid. Once a cell has gone through the process of meiosis, it can no longer function as an integral part of the parent body but is sooner or later extruded to serve in the formation of a new individual. In multicellular animals there exist two types of sex cells: the female cells or ova, and the male cells or spermatozoa. As a rule the two cells of the opposite sexes must unite in the process of fertilization before development can start. When the two gametes (the ovum and the spermatozoon) unite, they fuse into a single cell, the zygote, which again has a diploid number of chromosomes. The zygote, or fertilized ovum, then proceeds to develop into a new adult animal.

Side by side with sexual reproduction there exists in many species of animals a different mode of producing new generations—asexual reproduction. In asexual reproduction the offspring are not derived from generative cells (gametes) but rather from parts of the parent's body consisting of somatic cells. The size of the part which is set aside as the rudiment of the new individual may be large or small, but in the Metazoa it always consists of more than one cell. The development of an animal by way of asexual reproduction obviously belongs in the same category as the development from an egg and should be treated as a special form of ontogenetic development. It will be dealt with in Chapter 20. To distinguish between the two forms of ontogenetic development, the term **embryogenesis** may be used to denote development from the egg, and the term **blastogenesis** may be used for the development of new individuals by means of asexual reproduction.

1-2 THE PHASES OF ONTOGENETIC DEVELOPMENT

From what has already been said it is clear that the processes leading to the development of a new individual really start before the fertilization of the egg, because the ripening of the egg and the formation of the spermatozoon, which constitute the phase of gametogenesis, create the conditions from which the subsequent embryogenesis takes its start. In both oogenesis and spermatogenesis, meiosis, by discarding half of the chromosomes, singles out the set of genes which are to operate in the development of a particular individual. In both sexes the initial cells giving rise to the gametes are very similar and, as a rule, not essentially different from other cells of the body except that these cells are not involved in any of the differentiations serving to support the life of the parent individual. In both sexes the first step in the production of gametes is a more or less rapid proliferation of cells by ordinary mitosis. The proliferating cells in the testes are known as the spermatogonia; the proliferating cells in the ovaries are called oogonia. Once proliferation ceases, the cells are called spermatocytes in the male and oocytes in the female. They then enter into a stage of growth and later into a stage of maturation.

Although the stage of proliferation is not essentially different in the male and female, the processes of growth and maturation in the two sexes differ to a very great extent. The cytoplasmic differentiations of the spermatozoon enable it to reach the egg by active movement and to fertilize it. On the other hand, the egg cell accumulates in its cytoplasm substances which are used up during development—either directly, by becoming transformed into the various structures of which the embryo consists, or indirectly, as sources of energy for development. The elaboration in the egg cell of cytoplasmic substances to be used by the embryo and their placing in correct positions are essential parts of what occurs during the first phase of development.

The second phase of development is fertilization. Fertilization involves a number of rather independent biological and physiological processes. First, the spermatozoa

must be brought into proximity with the eggs if fertilization is to occur. This involves adaptations on the part of the parents which insure that they meet during the breeding season, discharge their sex cells simultaneously in cases of external fertilization, or copulate in cases of internal fertilization. Next, the spermatozoa must find the egg and penetrate into the egg cytoplasm. This entails a very finely adjusted mechanism of morphological and physicochemical reactions. The egg is then activated by a spermatozoon and starts developing. A further rearrangement of the organ-forming substances in the egg is among the first changes that take place in the egg after fertiliza-

tion. (See Section 5-6.) The third phase of development is the period of cleavage. The fertilized egg is still a single cell, since the nucleus and cytoplasm of the spermatozoon fuse with the nucleus and cytoplasm of the egg. If a complex and multicellular organism is to develop from a single cell, the egg, the latter must give rise to a large number of cells. This is achieved by a number of mitotic cell divisions following one another in quick successsion. During this period the size of the embryo does not change, the cleavage cells or blastomeres becoming smaller and smaller with each division. No far-reaching changes can be discovered in the substance of the developing embryo during the period of cleavage, as if the preoccupation with the increase of cell numbers excludes the possibility of any other activity. The whole process of cleavage is dominated by the cytoplasmic organoids of the cells, the centrosomes and achromatic figures. The nuclei multiply but do not interfere with the processes going on in the cytoplasm. The result of cleavage is sometimes a compact heap of cells, but usually the cells are arranged in a hollow spherical body, a blastula, with a layer of cells, the blastoderm, surrounding a cavity, the blastocoele.

The fourth phase of development, that of gastrulation, follows. During this phase the single layer of cells, the blastoderm, gives rise to two or more layers of cells known as the germinal layers. The germinal layers are complex rudiments from which are derived the various organs of the animal's body. In higher animals the body consists of several layers of tissues and organs, such as the skin, the subcutaneous connective tissue, the layer of muscles, the wall of the gut, and so on. All these tissues and organs may be traced back to three layers of cells—the aforementioned germinal layers. Of these the external one, the ectoderm, always gives rise to the skin epidermis and the nervous system. The layer next to the first, the mesoderm, is the source of the muscles, the blood vascular system, the lining of the secondary body cavity (the coelom, in animals in which such a cavity is present), and the sex organs. In many animals, particularly the vertebrates, the excretory system and most of the internal skeleton are also derived from the mesoderm. The third and innermost germinal layer, the endoderm,

forms the alimentary canal and the digestive glands.

The germinal layers are produced by the disappearance of a part of the blastoderm from the surface and its enclosure by the remainder of the blastoderm. The part that remains on the surface becomes ectoderm; the part disappearing into the interior becomes endoderm and mesoderm. The disappearance of endoderm and mesoderm from the surface sometimes takes the form of a folding-in of part of the blastoderm, so that the simple spherical body becomes converted into a double-walled cup, as if one side of the wall of an elastic hollow ball had been pushed in by an external force. This infolding or pushing in of the endoderm and mesoderm is known as **invagination**, and the resulting embryo is known as a **gastrula**—whence the term **gastrulation**. The way in which the endoderm and mesoderm become separated from each other in the interior of the gastrula varies a great deal in different animals and cannot be described in this general review. (See Chapter 7.) If the gastrula is formed by invagination, the cavity of the double-walled cup is called the **archenteron**, and the opening leading from

this cavity to the exterior is called the blastopore. In animals in which the gastrula is formed in a different way-not by invagination-the cavity (archenteron) and the

opening of the cavity to the exterior (blastopore) may still appear later on.

The archenteron, or part of it, eventually gives rise to the cavity of the alimentary system. The fate of the blastopore differs in the three main groups of Metazoa. In Coelenterata it becomes the oral opening. In Protostomia (including Annelida, Mollusca, Arthropoda, and allied groups) it becomes subdivided into two openings, one of which becomes the mouth and the other the anus. In Deuterostomia (including Echinodermata and Chordata) only the anal opening is connected in its development with the blastopore, the mouth being formed later on as an independent perforation of the body wall. The whole of the lining of the alimentary canal does not always consist of endoderm; in all groups of animals the ectoderm may be invaginated secondarily at the oral or at both oral and anal openings to become a part of the alimentary canal. The parts of the alimentary canal lined by ectoderm are known as the stomodeum (adjoining the mouth) and proctodeum (adjoining the anus).

With the formation of the three germinal layers, the process of subdivision of the embryo into parts with specific destinies commences. This subdivision is continued in the next (fifth) phase of development, the phase of organogenesis (organ formation). The continuous masses of cells of the three germinal layers become split up into smaller groups of cells, each of which is destined to produce a certain organ or part of the animal. Every organ begins its development as a group of cells segregated from the other cells of the embryo. This group of cells we will call the rudiment of the respective organ. The rudiments into which the germinal layers become subdivided are called primary organ rudiments. Some of these are very complex, containing cells destined to produce a whole system of organs, such as the entire nervous system or the alimentary canal. These complex primary organ rudiments later become subdivided into secondary organ rudiments-the rudiments of the subordinated and simpler organs and parts. The formation of the primary organ rudiments follows so closely on the processes of gastrulation that the two processes can hardly be considered separately. Dynamically they are linked into one whole and will be described in conjunction in the following pages. The chapters on organogenesis will then be concerned with the later development of the primary organ rudiments and with the formation of the secondary organ rudiments. With the appearance of primary and secondary organ rudiments, the embryo begins to show some similarity to the adult animal, or to the larva if the development includes a larval stage.

The sixth phase of development is the period of growth and histological differentiation. After the organ rudiments are formed they begin to grow and greatly increase their volume. In this way the animal gradually achieves the size of its parents. Sooner or later the cells in each rudiment become histologically differentiated; that is, they acquire the structure and physicochemical properties which enable them to perform their physiological functions. When the cells in all the organs, or at least in the vitally important organs, have become capable of performing their physiological functions, the young animal can embark upon an independent existence - an existence in which it has to procure food from the surrounding environment.

In rather rare cases (in the nematodes, for instance) the young animal emerging from the egg is a miniature copy of the adult animal and differs from the latter only in size and the degree of differentiation of the sex organs. In this case the subsequent development consists only of growth and maturation of the gonads. It is more usual, however, for animals emerging from eggs to differ from the adult to a greater extent; not only the gonads but also other organs may not be fully differentiated, or they may even be absent altogether and have to develop later. Sometimes the animal

emerging from the egg possesses special organs which are absent in the adult but which are necessary for the special mode of existence of the young animal. In this case the young animal is called a larva. The larva may lead a different mode of life from the adult, and therein lies one of the advantages of having a larval stage in development. The larva undergoes a process of metamorphosis when it is transformed into an animal similar to the adult. The metamorphosis involves more or less drastic changes in the organization of the larva, depending on the degree of difference between the larva and the adult. During metamorphosis new organs may develop, so that morphogenetic processes become active again after a more or less prolonged period of larval life.

A secondary activation of morphogenetic processes may be produced in a different way. Many animals possess considerable plasticity and may be able to repair injuries sustained from the environment or caused experimentally. Lost parts may be **regenerated**, and this means that the developmental processes may sometimes be repeated in an adult or adolescent organism.

Asexual reproduction of animals involves the development of new parts and or-

gans in animals that have already achieved the adult stage.

All morphogenetic processes occurring in the later life of the animal, after the larval stage, or even when the adult stage has been achieved, will be dealt with as constituting a seventh and last phase of development.

1-3 HISTORICAL REVIEW OF THE MAIN TRENDS OF THOUGHT IN EMBRYOLOGY*

Descriptive and Comparative Embryology. Although the correct understanding of ontogenetic development could be achieved only after the establishment of the cell theory, fragmentary information on the development of animals has been obtained since very ancient times. Aristotle had described the development of the chick in the egg as early as 340 B.C. Many observations on development of various animals, especially of insects and vertebrates, were made in the seventeenth and eighteenth centuries. However, the data of embryology were first presented in a coherent form by Karl Ernst von Baer (1828). In his book, Ueber Entwicklungsgeschiechte der Tiere, Beobachtung und Reflexion, Baer not only summed up the existing data and supplemented them by his original investigations but also made some important generalizations. The most important of these is known as Baer's law. The law can be formulated thus: "More general features that are common to all the members of a group of animals are, in the embryo, developed earlier than the more special features which distinguish the various members of the group." Thus the features that characterize all vertebrate animals (brain and spinal cord, axial skeleton in the form of a notochord, segmented muscles, aortic arches) are developed earlier than the features distinguishing the various classes of vertebrates (limbs in quadrupeds, hair in mammals, feathers in birds, etc.). The characters distinguishing the families, genera, and species come last in the development of the individual. The early embryo thus has a structure common to all members of a large group of the animal kingdom and may be said to represent the basic plan of organization of that particular group. The groups having a common basic plan of organization are the phyla of the animal kingdom.

Baer's law was formulated at a time when the theory of evolution was not recognized by the majority of biologists. It has been found, however, that the law can be

^{*}Further references: Nordenskiöld, 1929; Needham, Part II, 1931; Singer, 1931; Hall, 1951; Gabriel and Fogel, 1955; Needham, 1959; Oppenheimer, 1967.

reinterpreted in the light of the evolutionary theory. In its new form the law is known as the **biogenetic law** of Müller-Haeckel. Müller propounded the law in its new form and supported it by extensive observations on the development of crustaceans (1864). Haeckel (in 1868) gave it the name of the 'biogenetic law' and contributed most to its wide application in biology.

According to Baer's law, the common features of large groups of animals develop earliest during ontogeny. In the light of the evolutionary theory, however, these features are the ones that are inherited from the common ancestor of the animal group in question; therefore, they have an ancient origin. The features that distinguish the various animals from one another are those that the animals have acquired later in the course of their evolution. Baer's law states that these features in ontogeny develop at later stages. Briefly, the features of ancient origin develop early in ontogeny; features of newer origin develop late. Hence, the ontogenetic development presents the various features of the animal's organization in the same sequence as they evolved during the phylogenetic development. Ontogeny is a recapitulation of phylogeny.* The repetition is obviously not a complete one, and the biogenetic law states that "ontogeny is a shortened and modified recapitulation of phylogeny." The shortening of the process is evident not only from the fact that what had once taken thousands of millions of years (phylogeny) is now performed in a matter of days and weeks (ontogeny), but also from the fact that many stages which occurred in the original phylogenetic development may be omitted in ontogeny. The modifications arise mainly because the embryo at any given time is a living system which has to be in harmony with its surroundings if it is to stay alive. The embryo must be adapted to its surroundings, and these adaptations often necessitate the modification of inherited features of organization. A good example of such adaptation is the placenta in mammals. The placenta is a structure developed by the embryo to establish a connection with the uterine wall of the mother and thus to provide for the nutrition of the embryo. This structure, though developed rather early in the life of the embryo, could not have existed in the adult mammalian ancestors. It is obviously an adaptation to the special conditions in which a mammalian embryo develops.

Even if the repetition of features of their ancestors in the ontogenetic development of contemporary animals is not complete, the fragmentary repetition of certain ancestral characters may still be very useful in elucidating the relationships of animals. As an example of this we may consider the formation of gill clefts or, at least, pharyngeal pouches in the ontogenetic development of all vertebrates. In the aquatic vertebrates, such as Cyclostomata and fishes, the gill clefts serve as respiratory organs. In the adult state of terrestrial vertebrates, the pharyngeal pouches have disappeared completely or have been modified out of all recognition, and the function of respiration has been taken over by other organs—the lungs. Nevertheless, the pharyngeal pouches appear in the embryo. (See Figure 391.) In amphibians whose larvae are aquatic, the pharyngeal pouches at least temporarily serve for respiration. In reptiles, birds, and mammals, the pharyngeal pouches of the embryo do not serve for respiration at all. Their formation can be explained only as an indication that the terrestrial vertebrates have been derived from aquatic forms with functional gills. The paleontological evidence fully confirms this conclusion.

The systematic position of some animals cannot be recognized from adult structure, owing to profound modification acquired as a result of adaptation to very special conditions. Here the knowledge of the development sometimes throws unexpected

^{*}For a modern explanation of the facts on which the biogenetic law is based, see page 518.

light on true relationships. The adult ascidian is a sessile animal with no organs of locomotion and a nervous system of a very primitive nature. The adult animal had been classed as a near relative of molluscs until Kowaleysky (1866) discovered that the larvae of the ascidians possess a well-developed dorsal brain and spinal cord, a definite notochord, and lateral bands of muscles (in short, organs that are typical for the vertebrates). The ascidians are therefore considered as belonging to the same phylum as the vertebrates, the phylum Chordata (Fig. 1).

In the adult parasitic animal Sacculina, the organization of the animal is very much simplified in relation to the easy life that the parasite enjoys; it is reduced practically to a shapeless sack producing eggs and a system of branched rhizoids, by means of which the parasite is attached to its host, the crab, and absorbs the host's body fluids on which it feeds. It would be impossible to place Sacculina in any group of the animal kingdom if its development were not known (Fig. 2). However, the larva of Sacculina is a typical arthropod, bearing a close similarity to the larvae of the lower crustaceans, the Entomostraca (Delage, 1884).

A rather similar larva is also found in the barnacles (Cirripedia) which, though possessing jointed legs like other arthropods, have lost the segmentation of the body in the

adult state.

The attachment of the starfish larva, the brachiolaria, to the substrate while it is metamorphosing into the definitive form is an indication that the free-living echinoderms have been derived from sessile forms. This conclusion is again borne out by the evidence of paleontology.

Following the principles of Baer's law and the biogenetic law, embryologists have systematically investigated the development of animals belonging to all the major

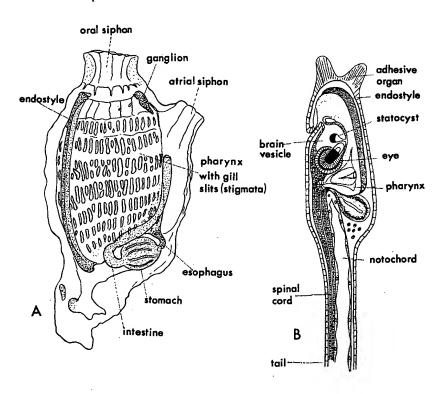


Figure 1. A, Adult ascidian, Ciona intestinalis. B, Larva of ascidian, lateral view. (After Kowalevsky, from Korschelt, 1936.)

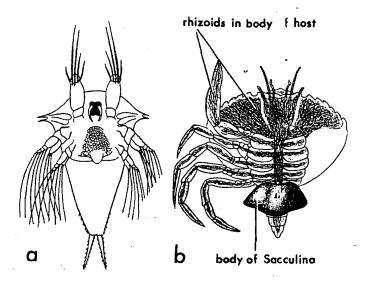


Figure 2. Parasitic cirripede, Sacculina. Nauplius larva (a) and adult (b) attached to a crab. (After Delage, from Parker and Haswell. Text-book of Zoology, Macmillan, London, and from Korschelt, 1936.)

groups of the animal kingdom. As a result of very extensive and painstaking investigations, a magnificent edifice of comparative embryology has been built.

Explaining Development: Theories of Preformation and Epigenesis. Neither the description of morphological transformations occurring in the embryo nor the comparison of embryos and larvae among themselves and with the adult animals exhausts all the problems presented by the ontogenetic development of animals. The fundamental problem presented by the existence of cyclical ontogenetic changes is the question: Why does ontogeny occur at all? What are the forces which produce the changes? How is it that, starting from a simple spherical cell, the process always ends in producing a highly complex and specific structure which, though varying in detail, reproduces with astonishing perseverance the same or almost the same adult form?

Attempts at solving this basic problem have been made ever since the human mind recognized the existence of development. For a long time the explanations proposed were purely speculative. Aristotle attempted to give a solution to the problem of ontogeny along the general lines of his philosophical teaching, distinguishing between the **substance** and the **form** of things. The form appears in this concept as the creative principle. Aristotle further supposed that the substance for the development of a child is provided by the mother (in the form of nutrition) but that the creative principle is supplied by the father. He thus accounted also for the necessity of fertilization. Although this treatment of the phenomena of development is completely contradictory to what we now know of the material basis of development (the parts played by the ovum and the spermatozoon), still the concept of a creative principle has turned up repeatedly in the teachings of embryologists up to the twentieth century.

In the seventeenth and eighteenth centuries, when all biological sciences developed rapidly, together with the physicochemical sciences, there existed a widespread theory explaining the ontogenetic development of animals. This was the **theory of preformation**. The theory of preformation claimed that if we see that something develops from the egg, then this something must actually have been there all the time but in an invisible form. It is common knowledge that in a bud of a tree the leaves, and sometimes also the flowers with all their parts, can be discovered long before the bud starts growing and spreading and thus exposing to view all that before was hidden inside, covered by the superficial scales of the bud. Furthermore, it was known that in a chrysalis of a butterfly the parts of the butterfly's body—the legs, the wings, etc.—can be

discovered if the cuticular coat of the chrysalis is carefully removed a few days before the butterfly emerges from the chrysalis. Something of this sort was supposed to exist in the egg. All the parts of the future embryo were imagined to be already in the egg, but they were thought to be transparent, foided together, and very small, so that they could not be seen. When the embryo began to develop, these parts supposedly started to grow, unfold, and stretch themselves, become denser and therefore more readily visible. The embryo, and therefore indirectly also the future animal, was **preformed** in

the egg. Hence the theory is called the theory of preformation.

When spermatozoa were discovered in the seminal fluid, the relative significance of the ova and spermatozoa had to be accounted for. It is obvious that a preformed embryo cannot be present in both the egg and the spermatozoon. The preformationists were split therefore into two rival schools, the ovists and the animalculists. (The latter name comes from the word animalcule, as the spermatozoa were then called.) The ovists asserted that the embryo was preformed in the egg. The spermatozoa then seemed superfluous, and in fact, they were declared parasites living in the spermatic fluid. On the other hand, the animalculists declared that the embryo was preformed in the spermatozoon and that the egg served only to supply nutrition for the developing embryo. A lively discussion arose, which ended in favor of the ovists. The victory of the ovists was due to the discovery of parthenogenetic development in some insects, e.g., the aphids (Bonnet, 1745). If the egg could develop without fertilization it was clear that the embryo could not be preformed in the spermatozoon.

The theory of preformation, although very popular in its time, did not satisfy all biologists, and opposing views, denying the existence of a preformed embryo in the egg, were proposed. The most important contribution in this field was the theory of epigenesis, proposed by Caspar Friedrich Wolff (1759). In favor of his theory Wolff adduced his own observations on the formation of the chick embryo. In the earliest stages of the development of the chick he could not find any parts of the future embryo. Moreover, he found that the egg was by no means devoid of any visible structure; there was a structure present, but it was different from that of the later embryo. Wolff found that the substance of which the embryo is composed is granular. Presumably the granules must have been the cells or their nuclei. These granules were later arranged into the layers which we now call the germinal layers. Wolff saw that by the formation of local thickenings in some parts of these layers, by thinning out in others, and by the formation of folds and pockets, the layers are transformed into the body of the embryo. He concluded, therefore, that in the early egg there does not exist a preformed embryo but only the material of which the embryo is built. This material does not represent an embryo any more than a heap of bricks represents the house that will be built of them. In both cases there had to be an architect who would use the material for a purpose that he had in mind. In the case of the developing embryo the architect was represented by a vital force, perhaps not essentially different from the "creative principle" postulated by Aristotle.

Experimental Embryology. Wolff's observations, however, could not be considered as final in deciding between the alternative theories of preformation and epigenesis. In spite of what he actually observed, it was still conceivable that organs and parts of the body of the future embryo were represented in the egg by discrete particles, qualitatively different among themselves. The granules which he saw might have been different in their properties. Even if the transformation of such qualitatively different parts into the organs of the embryo should have been more complicated than was envisaged by the crude preformistic theory, the principle of preformation might well have held true in spite of the apparent homogeneity of the material of which the embryo was supposedly made. Observation alone could not make further advances toward

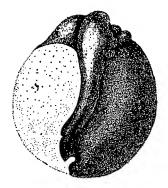


Figure 3. Half embryo produced by W. Roux by killing one blastomere of a frog's egg with a hot needle. (After Roux, from Morgan, 1927.)

the solution of this problem, and further progress could be achieved only with the aid of experiment.

One of the experiments which is relevant to the preceding problem is the separation of the two cells into which the egg is divided at the beginning of development. If the theory of preformation is correct, we should expect that one of the two first blastomeres, containing one half of the egg material, should develop into an embryo lacking one half of its organs and parts. If, on the other hand, the substances contained in the egg are but the building material used for the construction of the embryo, then it is conceivable that half of the material might be sufficient for making a complete embryo even if it may have to be on a diminished scale, just as the bricks prepared for the construction of a big house may be used for building two houses of a smaller size.

The first embryologist to see this way of solving the problem was Wilhelm Roux (1850-1924). Accordingly, he proceeded to test one of the first two cleavage cells in the common frog for its ability to develop. To achieve his end Roux destroyed one of the two cleavage cells with a red-hot needle (1888). The embryo that was derived from the surviving cleavage cell was found to develop, at least at first, as if it were still forming a half of a complete embryo. In other words, the developing embryo was defective, as it should have been according to the theory of preformation (Fig. 3). It was found later, however, that the technique used by Roux was too crude. The damaged cleavage cell had not been removed, and it was the presence of this damaged cleavage cell, as was later found out, that caused the defects in the surviving embryo. If the two cleavage cells of the egg were separated completely, two whole and, except for their size, normal embryos could develop, one from each of the two cleavage cells. This result was first found by H. Driesch (1891), working on sea urchin eggs (Fig. 4), and later by Endres (1895) and Spemann (1901, 1903), working with eggs of newts. Eventually the experiment was repeated by Schmidt (1933) on the frog, the same animal that had served for the experiments of Roux. Schmidt found that if the two cleavage cells were completely separated, each could develop into a whole embryo (Fig. 5).

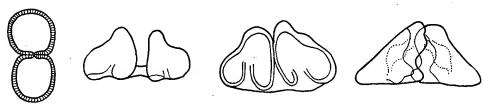
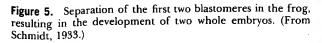
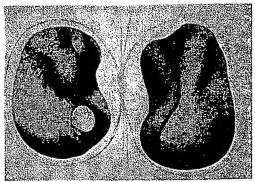


Figure 4. Separation of the first two blastomeres in the sea urchin, resulting in the development of two whole embryos. (After Driesch, from Gabriel and Fogel, 1955.)





The first experiments on the developing embryo were followed by many others, and soon a new science was born: experimental embryology (Roux, 1905).

Experimental embryology, in contrast to comparative embryology or descriptive embryology, uses experiment as a method of investigation. However, the use of the experimental method in itself does not create a science or a branch of science. New branches of science are created by novel viewpoints and novel problems set before science. It was the problem of what ontogenetic development actually is, what the driving forces behind it are, that necessitated the application of experiment after the methods of speculation and of pure observation were found to be impotent in solving the problem.

Modern Embryology - Analytical Embryology. After the middle of the present century embryology had got caught up in the new trend that developed in biological science. Early in the century, the background for this new trend was established mainly by the work of T. H. Morgan and his school (Morgan, 1919). This work proved that the units of heredity, the genes, are arranged in linear order in the chromosomes of the cells. Analysis shows that chromosomes consist of several chemical components: deoxyri-

bonucleic acid (DNA), ribonucleic acid (RNA), and proteins.

In an epoch-making paper published in 1953, Watson and Crick suggested that the deoxyribonucleic acid, as found in the chromosomes, consists of pairs of very elongated molecules twisted spirally around each other in a double helix. Each strand of the helix is made up of a number of units, the mononucleotides, which differ from one another only in the nitrogenous base (i.e., adenine, thymine, guanine, or cytosine) which each contains. The bases form two pairs, which structurally "fit" together, so that in the intertwined double helix adenine always links with thymine, and guanine with cytosine. Further work made it clear that the arrangement of the bases in the molecule of deoxyribonucleic acid contains a code for the proteins that may be synthesized by a particular species of organism. The code is essentially a series of "triplets" - groups of three bases which correspond to one amino acid in a polypeptide (protein) chain. Thus, a sequence of triplets in the DNA determines a sequence of amino acids in a protein molecule, and the section of the deoxyribonucleic acid molecule containing this sequence is the essential part of what geneticists call a "gene." The "genetic code," showing which sequences of bases correspond to which of the 20 amino acids constituting most of the proteins in the organic world, is shown in Table 1. Note that several different triplets in the DNA may code for the same amino acid.

Between the genetic code in the chromosomal DNA and the cell proteins, there are certain intermediate steps. The "message" contained in the DNA must first be copied in the form of a ribonucleic acid molecule, whose nucleotide sequence is complementary to the nucleotide sequency of the DNA (except that uridine takes the place of

TABLE 1 The Genetic Code

| 2nd Letter 1st Letter | U (Uracil*) | C (Ĉytosine) | A (Adenine) | G (Guanine) |
|--------------------------------|---|--------------------------|---|---|
| U (Uracil*) | UUU Phenylalanine UUA UUA UUG | UCU UCC UCA UCG | UAU UAC UAA Termination UAG of code of one gene | UGU Cysteine UGA Termination of code of one gene UGG tryptophan |
| C (Cytosine) | CUU CUC CAU CUG | CCU CCC CCA CCG | CAU CAC CAA CAG CAG | CGU CGC CGA CGG |
| A (Adenine) | AUU AUC Isoleucine AUA AUG Methionine** | ACU ACC ACA ACG | AAU AAC AAA AAG Lysine | AGU Serine AGC AGA Arginine |
| G (Guanine) | GUU GUC GUA Valine or Methionine | GCU GCC GCA GCG | GAU GAC GAA GAG GAG | GGU GGC GGA GGG |

*Uracil in ribonucleic acids corresponds to thymine in deoxyribonucleic acids.

**The methionine serves for initiation of a polypeptide chain. In bacteria, the amino acid is formylmethionine. In both cases AUG serves as the beginning of a genetic message (gene).

After M. Nirenberg and collaborators and H. G. Khorana and collaborators; see Proc. Nat. Acad. Sc. U.S.A., Vol. 54, 1965, pp. 954-960 and 1378-1385.

thymine). This is the "transcription" phase. The code is then contained in an RNA molecule ("messenger" RNA). Two further kinds of ribonucleic acid are modeled on the DNA: the ribosomal RNA, which together with certain proteins forms small (±200 Å in diameter) particles, the **ribosomes**; and the transfer RNA, which is involved in bringing the correct amino acid to the ribosome, where the amino acids become arranged and joined together in the correct sequence according to the code contained in the messenger RNA. This procedure constitutes the "translation" phase.

The importance of these discoveries for embryology derives from the following considerations. It has become evident that all the properties of any organism are determined in the last instance by the sequence of base triplets in the DNA molecules. Furthermore, it is accepted that the sequence of the base triplets directly determines what kinds of proteins can be produced by an organism. All other manifestations specific to any organism, whether morphological or physiological, depend more or less directly on the assortment of proteins coded for by the hereditary DNA. This new way of looking at the organic world shifts the problem of ontogenetic development directly into the realm of molecular relationships. It also makes possible, in principle, the construction of a complete theory of development. Such a theory would start with the triplet sequences in the DNA and would show first how these sequences are "read out" by transforming them into an array of proteins, placed and distributed in an organized way in

space and time, and then would show how the proteins, acting partly on their own and partly through other chemical components, produce the complicated system that is

an adult organism (animal, in the context of this book).

A whole array of new techniques has been mobilized in working toward such a theory of development. Electron microscopy has made great advances after the mid-1950's, when methods were developed for embedding tissues in plastics and for cutting ultrathin sections for the study of the fine structure of cells. Refined methods of chemical analysis, such as chromatography, electrophoresis, ultracentrifugation, and the use of radioactive tracers, have been put at the disposal of embryologists.

With the change in the theoretical background and techniques, a subtle change has permeated the work of embryologists. The aim of investigation is no longer the study of the development of any particular animal, or any group of animals, but the discovery of the basic principles and processes of development. This trend in science may perhaps be called **analytical embryology**, and this is what "modern" embryology ac-

tually is.

It must be realized that analytical embryology can proceed only on the basis of knowledge provided by descriptive embryology, because after all, it is the actual course of the transformations that have to be explained by the theory of development, of comparative embryology, because it is necessary to know of how general a significance any particular phenomenon of development is, and of experimental embryology, because it has revealed the causal relationships of many developmental processes.

In this "introduction to embryology" all the approaches to development (i.e., the descriptive and the comparative embryology and the experimental and the analytical

embryology) will be dealt with together as far as possible.